

Rapamycin reduces *Drosophila* longevity under low nutrition

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ABSTRACT : Rapamycin treatment is considered a pharmacological intervention with the potential to mimic the longevity benefits of dietary manipulations. However, how rapamycin interacts with nutrition is not fully understood. Here we studied the effect of rapamycin on the longevity of *Drosophila* under a range of dietary conditions. In diets low in nutrients, rapamycin reduced longevity in a dosage-dependent manner. This dosage effect requires some nutrients as rapamycin has no impact on survival under starvation conditions. Under a balanced diet of yeast and sugar, rapamycin had no repeatable beneficial effect on organismal longevity. These results show that the effect of rapamycin on longevity is sensitive to the nutritional environment and it can reduce lifespan when nutrients are limited.

KEYWORDS: Nutrition, Longevity, Rapamycin

I. INTRODUCTION

Studies in mammals and invertebrates have identified rapamycin as a drug that may mimic the benefits of diet restriction and have anti-aging effects (1–8). Rapamycin inhibits the TORC1 and TORC2 complexes of the mammalian target of rapamycin (mTOR) pathway that regulates nutrient sensing, placing rapamycin at the interface between nutrition and longevity control (1–7). The promise of rapamycin as a pharmacological agent that can extend longevity calls for a thorough understanding of the molecular targets and environmental mediators of rapamycin's effect on longevity. In genetically heterogeneous mice, rapamycin treatment can extend the life span of both males and females (9,10). In flies, two independent studies reported longevity effects of rapamycin (11,12). The most comprehensive of those studies showed increased lifespan of flies maintained on food supplemented with concentrations of rapamycin ranging from 50 μM to 400 μM. At a fixed concentration of 200 μM, a concentration at which flies are expected to incorporate a similar amount of rapamycin as that reported in studies of mice, rapamycin extended longevity in several diets (12). Independently, the second study reported that flies maintained on a nutrient rich food supplemented with 1, 10 or 100 μM of rapamycin showed no significant differences in life span, but that rapamycin could have detrimental effects at 500 μM or higher concentrations (11). Here we undertook an independent study of the effect of rapamycin on *Drosophila* longevity under a range of dietary conditions.

Since amino acid imbalance is a key determinant of *Drosophila* lifespan (13), the restriction of food in *Drosophila* is frequently performed via the dilution of yeast as a protein source. Here we measured the effect of rapamycin on longevity of *Drosophila* maintained on four different diets with a range of yeast levels (1, 2, 3, 4 and 8 and 12% weight/volume) spanning the conditions that extend longevity in previous experiments (14). We further examined the effects of rapamycin on survival on a starvation diet (0.8% w/v agar in water with no additional nutrients). Rapamycin was added to the diets at different concentrations to explore both dose-dependent and presence-absence effects.

II. MATERIALS AND METHODS

All stocks were maintained and conducted under standard conditions (25 °C, 12 h light:12 h dark) on normal media (2% yeast, 11% sugar, 5.2% cornmeal, agar 0.8% w/v in water and 0.2% tegosept -methyl 4-hydroxybenzoate, from Sigma- St. Louis, MO, USA). All stocks were density controlled in replicate bottles using 48-hour egg lays by 20 pairs of parents for two generations prior to collection of flies for experimental assays. Adults were mated for 24 hours, anesthetized with moist CO₂, sorted by sex and allowed to recover for 24 hours before initiating longevity cages. 100 females were transferred to 1 liter demography cages. Dead flies were recorded and removed every 2 or 3 days, at which time fresh food was provided in a vial with 5ml of medium. We used three replicate cages for each treatment/genotype. For starvation studies flies were maintained in a 0.8% Difcobacto-agar in water. 0.1XSY food was prepared as described in (12). Rapamycin was purchased from LC Laboratories (Woburn, MA, USA.). Rapamycin was dissolved in ethanol and added to food, as described in (12).

III. RESULTS

3.1. Longevity effects of rapamycin under different diets

As expected, dietary restriction of yeast at 2, 3, or 4% diet causes a repeatable extension of longevity relative to 1% yeast, in two independent experimental blocks separated by 6 months (Figure 1, A and B). However, the addition of 200 μ M rapamycin to these diets does not show a consistent effect on longevity. On a diet of 2% yeast rapamycin extended longevity in one experiment (log rank test: $\chi^2 = 43.9$; $P < 0.0001$) but had no effect when repeated, resulting in a highly significant block effect in a proportional hazards model testing for main effects of rapamycin and block ($\chi^2 = 43.3$; $P < 0.0001$; see supplementary Table 1). On diets of 3% and 4% yeast the effects of rapamycin were reversed across repeat experiments, with one block showing non significant effects of rapamycin on these two diets and the second block showing significant effects in the opposite directions (see Figure 1 C and D; log rank test on 3% yeast: $\chi^2 = 5.91$, $P < 0.015$; on 4% yeast $\chi^2 = 11.15$, $P < 0.0008$). In a proportional hazards model, these data showed no main effect of rapamycin or block, but a highly significant rapamycin X block interaction (supplementary Table 1).

Interestingly, in a diet low in nutrients (1% yeast), rapamycin consistently decreased the mean lifespan in both experimental blocks by 28% and 16.5% respectively (see Figure 1 A-D; log rank test for 1% yeast: $\chi^2 = 54.09$, $P < 0.0001$; $\chi^2 = 13.49$, $P < 0.0002$ for block 1 and 2 (Figure 1C and D), respectively). A proportional hazards model showed a highly significant effect of rapamycin, no effect of block and a weak rapamycin X block interaction ($P < 0.0151$) that does not withstand correction for multiple tests for these four diets (Figure 1, and supplemental Table 1). Supplementary Table 1 presents a summary of the rapamycin and repeat-experiment (block) effects for each diet condition (1, 2, 3, and 4% yeast), as revealed by analysis of variance. The 1% yeast diet is unique in that it is the one condition that shows a highly significant main effect of rapamycin that reduces longevity coupled with no significant main effect of block (repeat experiments are consistent). The other diet conditions, ranging from 2 to 4% yeast, either show no significant main effect of rapamycin, or significant main effects of block, implying that the rapamycin effects are not repeatable. In an effort to determine if the longevity-extending effect of rapamycin were restricted to high-nutrient diets, we conducted a third independent longevity assay using 2% and 8% yeast. The 2% treatment was used to provide a reference to the other experimental blocks, since block effects are common in longevity assays. Rapamycin significantly extended the longevity of flies fed both 2% and 8% yeast in this third experiment, (log rank test for 2% yeast: $\chi^2 = 40.663$, $p < 0.001$], for 8% yeast: [$\chi^2 = 24.475$, $p < 0.001$]). Analysis of variance for the effects of rapamycin and yeast showed a significant effect of rapamycin treatment in both diets and a modest effect of the yeast diet (ANOVA: treatment [DF: 1, F: 42.332, $p < 0.001$], yeast [DF: 1, F: 4.116, $p = 0.043$] and yeast*treatment interaction [DF: 1, F: 0.165, $p = 0.685$]) (Figure 1E-F). It should be noted that the mean longevity in this third experiment were higher than in other experimental blocks, but the expected effect of somewhat reduced longevity under richer diet is observed (8% shows shorter longevity than 2% yeast; see Figure 1E-F). The longevity of the OreR strain of *D. melanogaster* is known to be rather unresponsive to diet in the 5-15% range on SY media, relative to other genotypes (15).

We next conducted a fourth independent study using increasing concentrations of rapamycin (0, 5, 50, 100, 200 and 400 μ M) in 2% and 12% yeast diet (Figure 2). Concentrations of rapamycin ranging from 50 to 200 μ M extended the mean longevity of flies fed 2% yeast diet, reaching maximum significant extension at 200 μ M rapamycin (log rank test for 2% yeast at 200 μ M $\chi^2 = 6.435$, $p < 0.01$) and decreasing at 400 μ M (Figure 4A and C). However, increasing concentrations of rapamycin were not able to extend longevity of flies fed a 12% yeast diet (Figure 2B and C) suggesting that higher concentrations of rapamycin are needed to extend the longevity of flies fed a very high yeast diet. Although there was not a significant difference in the longevity of the flies fed the yeast diets at 0 μ M rapamycin, analysis of variance for the effects of rapamycin and yeast showed a significant effect of the yeast diet (ANOVA: diet [DF: 1, F: 8.911, $p < 0.003$]) and yeast*treatment interaction (ANOVA [DF: 1, F: 3.31, $p = 0.006$]).

One other thing to note is the more linear shape of the survivorship curves in Figure 2 compared to Figure 1. Since the longevity measurement represented in figure 2 was performed at a different laboratory, we attribute this difference to a genotype X environment interaction (GxE) (16).

3.2. Longevity effects of rapamycin under low nutrition diets.

We hypothesized that low-nutrient diets might require lower concentrations of rapamycin to obtain the beneficial effects on longevity. To test this hypothesis we measured lifespan on 1% yeast using two standard laboratory strains (*w^{Dha}* and *OreR*) and rapamycin concentrations ranging from 5 to 200 μ M. At 5 μ M, rapamycin decreased median lifespan of *w^{Dha}* strain but did not significantly affect the lifespan of *OreR*, relative to the 0 μ M control. At higher concentrations, rapamycin decreased the life span of both strains in a dose-dependent manner (Figure 3C). Previous studies reported an extension of longevity by rapamycin in all diets tested, even in those with low nutritional value (12). Because the low nutritional value diet used in that study differs from the diet

used in our experiments, we repeated the survival analysis using the same low nutritional diet as published by Bjedov et al. 2010 (0.1X SY,(12)). Rapamycin significantly shortened the mean lifespan of both strains when maintained in a 0.1X SY diet (Figure 3D,E).

3.3.The effect of rapamycin on starvation resistance

We also studied the effect of rapamycin under starvation conditions. w^{Dha} and *OreR* strains maintained in a starvation diet were exposed to 5, 50 and 200 μ M of rapamycin. Rapamycin did not have any effect on the resistance to starvation of w^{Dha} and *OreR* flies (Figure 4A, B). Interestingly, w^{Dha} strain showed an overall higher resistance to starvation than *OreR* strain (Figure 4C). Several technical points are worth noting for these starvation assays. Bjedov et al (2010) used 1.5% agar in starvation assays – twice the concentration of our assay – and flies survived 1-2 days longer in their study than what we report here, suggesting that agar may have some nutritional value for *Drosophila*. Rapamycin has low solubility in water (17), and the amount of rapamycin delivered to flies in the no-nutrient agar-water medium may be lower than in media containing nutrients that could act as co-solvents for rapamycin delivery. However, all rapamycin exposures used ethanol as a co-solvent to enhance solubility(17).

IV. CONCLUSION

In summary, there are three conclusions from these experiments: 1) Under limited nutrition, rapamycin decreases the longevity of two *Drosophila* strains in a dosage-dependent manner (Figure 3) in two independent low-nutrition diets (1% yeast and 0.1X SY food). 2) Rapamycin does not consistently extend longevity of *Drosophilamelanogaster* under unrestricted diets (Figure 1 and 2). 3) The detrimental effects of rapamycin are not observed in two *Drosophila* strains when under starvation conditions (Figure 4). The lack of a detrimental effect of rapamycin under starvation suggests that the pharmacological effects of rapamycin depend upon the nutritional state of the fly. Thus, as a dietary restriction mimetic, the addition of rapamycin to a diet that is already low in nutrients may exacerbate malnutrition and reduce longevity. It may be that decreased longevity is not observed under starvation because rapamycin cannot further restrict dietary nutrients. However, rapamycin may need more time than only a few days to modulate longevity and its effect under starvation might be hindered by the short longevity of starved flies.

Although we did find that rapamycin can extend longevity at balanced diets with non-limiting nutritional conditions (Figure 1), these effects were not repeatable in different experimental blocks. Of the seven longevity experiments conducted on yeast diets in the 2-4% range, rapamycin extended longevity in three experiments, had no effect in three experiments, and reduced longevity in one experiment (Figure 1). These data suggest that longevity extension by rapamycin under particular diets is not sufficiently repeatable that it can over-ride variable environmental conditions that are typically associated with replication of longevity experiments at different times, even using identical nutrient conditions.

Environmental interactions in rapamycin treatment might also explain the different results described in the literature (11,12). Rapamycin strongly reduces female fecundity (12), and reproductive output typically shows a negative correlation (trade-off) with longevity. The variable evidence for longevity extension by rapamycin in *Drosophila* may be partly attributable to indirect effects on longevity due to variable inhibition of reproductive effort when nutrients are sufficient to support egg production. The detrimental effects of rapamycin under low nutrients that we describe here may be heightened if the nutrient level is sufficiently low that egg production is already turned off, preventing any further beneficial effect of rapamycin from its inhibitory impact on reproduction. While this trade-off hypothesis is plausible, it remains possible that the distinct results from different experiments represent subtle variation in nutrient levels that confound the delivery or metabolism of rapamycin upstream or downstream of its site of action.

While rapamycin may indeed be a promising drug for the treatment of age related diseases or the extension of lifespan, the complex interactions reported here suggest that the precise nutritional and environmental conditions under which rapamycin may realize this promise are not fully understood. There is ongoing controversy in the field about the generality of the effects of rapamycin (18,19), and it is incumbent on researchers in this field to resolve this uncertainty through independent, replicated experiments. Given the variable results from model organism studies conducted in different laboratories with standard diets, temperatures and growth conditions, the use of rapamycin to treat human ailments will be challenging given the widely varying dietary and environmental conditions to which the general public are exposed. With additional studies of how diet may modify the effects of rapamycin, these challenges may well be overcome.

V. ACKNOWLEDGEMENTS

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7. Figure Legends

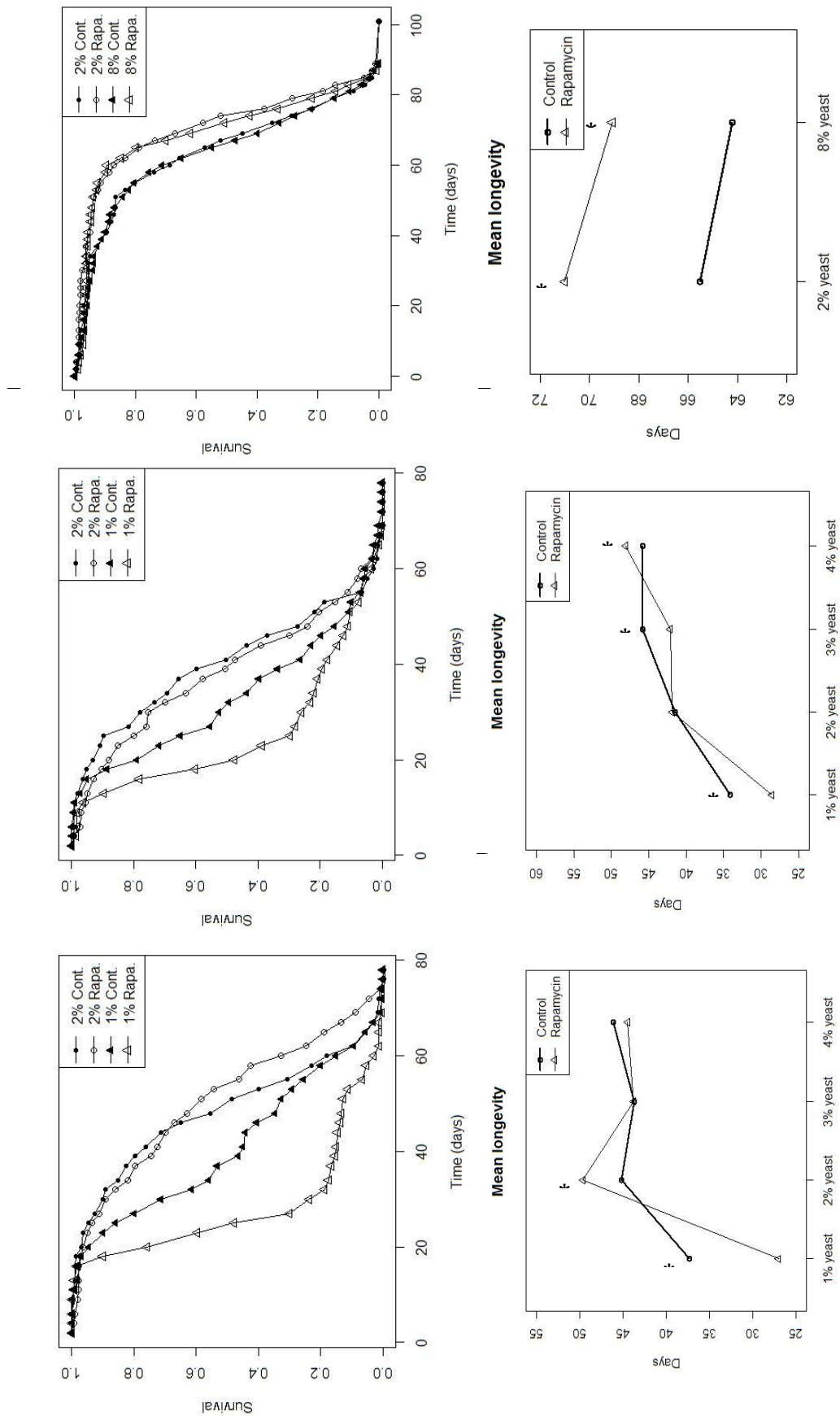


Figure 1. Longevity effects of rapamycin under different diets. Longevity analysis of *D. melanogaster* strain OreR under 2% (circle) and 1% yeast food (triangle) exposed to rapamycin (open symbols) and vehicle control (filled symbols). Panels A and B show results from two independent experiments, block 1 and 2, respectively. (C and D) Mean longevity of flies under rapamycin treatment (triangle) and control (circle) in 1, 2, 3 and 4% yeast food. Experimental block 1 (C) and block 2 (D) (E) Survival analysis of *D. melanogaster* OreR strain under 8% yeast food with 200 μ M rapamycin added. (F) Mean longevity values for the data in (E). * $P < 0.05$ log rank test.

Panel		Mean			% life span extension		
		A	B	E	A	B	E
1% yeast	Control	37.3	34.1				
	Rapa	27.9	28.5		-28%	16.4%	
2% yeast	Control	43.3	41.4	65.5			
	Rapa	45.6	41.8	71	5.1%		8.4%
3 % yeast	Control	44.7	45.7				
	Rapa	42.9	42.1			4%	
4 % yeast	Control	45.9	45.8				
	Rapa	46.1111	47.9			4.5%	
8% yeast	Control			64.2			
	Rapa			69			7.4%

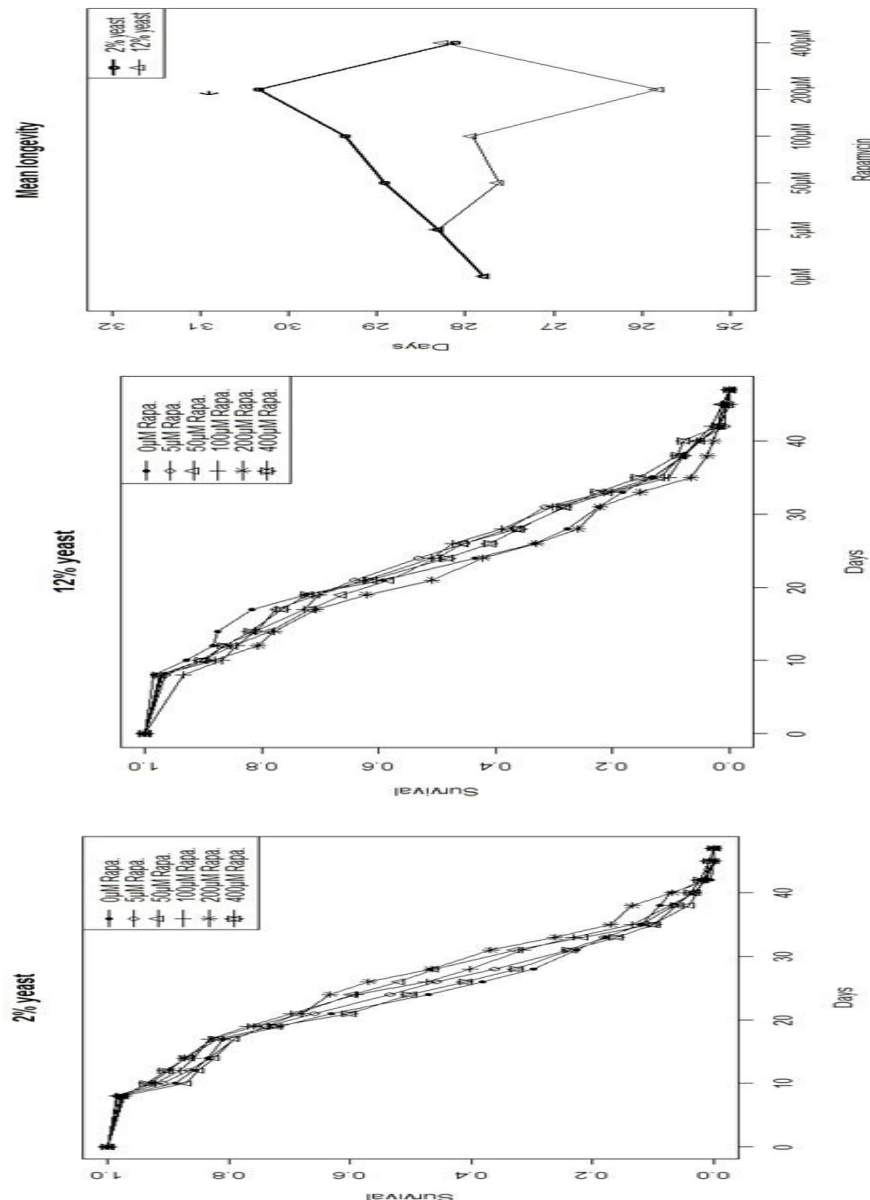


Figure 2. The longevity effect of different rapamycin concentrations.

Longevity analysis of *D. melanogaster* strain w^{Dha} under 2% (A) and 12% (B) yeast food exposed to 0, 5, 50, 100, 200 and 400 μ M. (C) Mean longevity for the data in A and B. * $P < 0.05$ log rank test for the comparison between 0 and 200 μ M.

	Mean		% life span extension	
	2% yeast	12% yeast	2% yeast	12% yeast
0 μ M	27.8	27.8		
5 μ M	28.3	28.3		
50 μ M	28.9	27.6	4	
100 μ M	29.4	27.9	5.7	
200 μ M	30.3	25.8	9	-7.2
400 μ M	28.2	28.2		

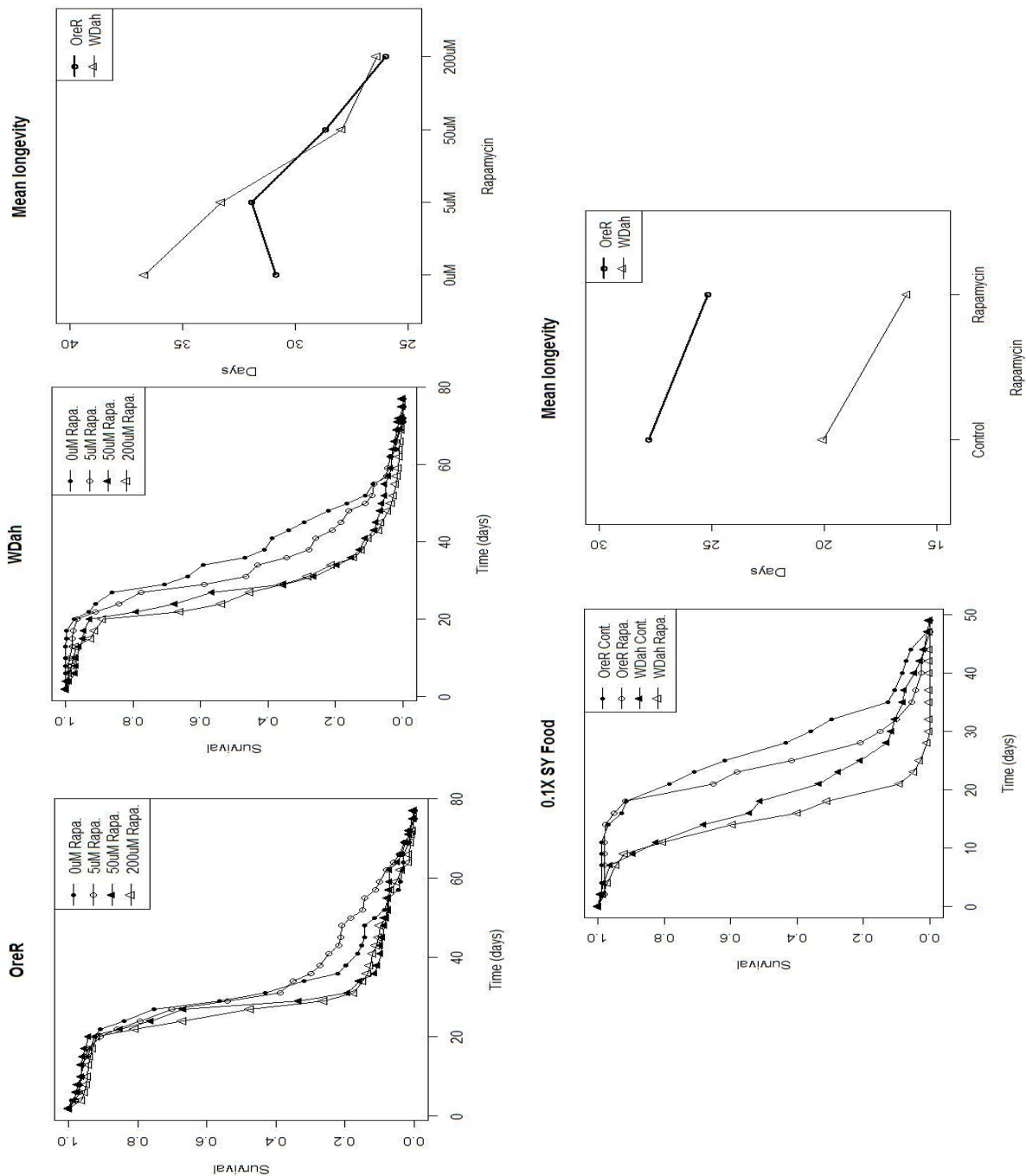


Figure 3. Longevity effects of rapamycin under low nutrition diets. Survival analysis of *D. melanogaster* strains *OreR* (A) and *w^{Dha}* (B) on 1% yeast food with 0, 5, 50 and 200 μ M rapamycin added. (C) Mean longevity for the data in A and B. (D) Survival analysis of *OreR* and *w^{Dha}* strains in 0.1X SY food with 200 μ M rapamycin added. (E) Mean longevity values for the data in (D). * $P < 0.05$ log rank test.

		Mean		% life span extension	
		OreR	WDah	OreR	WDah
0 uM		30.8	36.7		
5uM		31.9	33.2	3.5	-9.5
50uM		28.65	27.9	-4.5	-23.9
200uM		26	26.36	-15.5	-28.2
0.1SY OreR	Control	27.8175	20.0316		
	Rapa	25.1711	16.3152	-9.3	-18.5

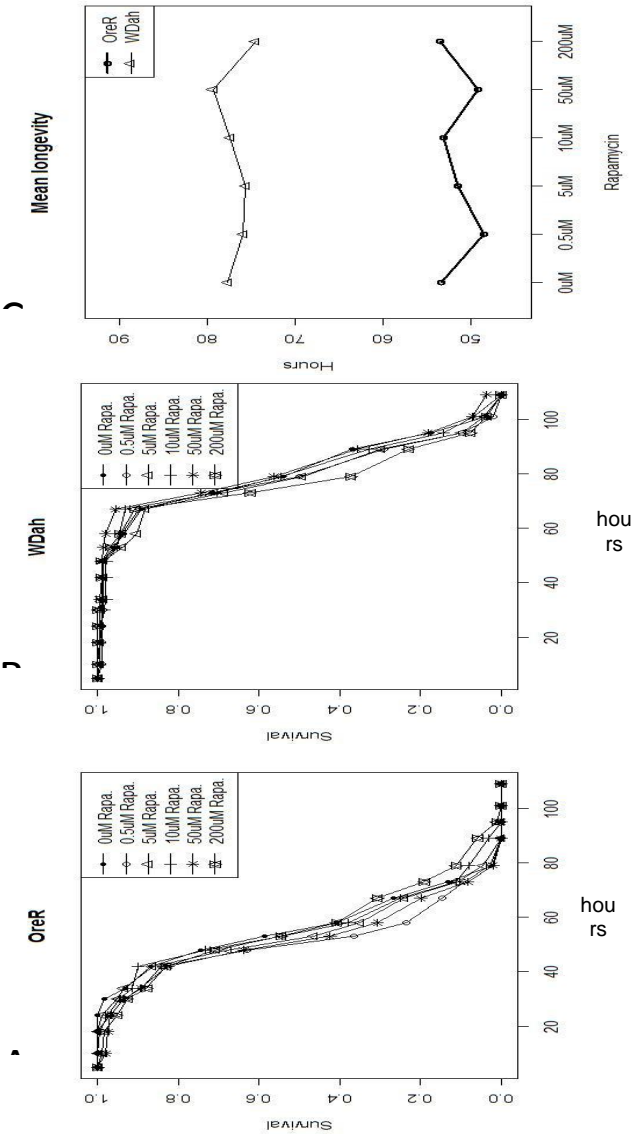


Figure 4. The effect of rapamycin on starvation resistance. Survival analysis of *D. melanogaster* OreR (A) and *w^{Dha}* (B) strains under starvation (0.8% agar in water) with 0.5, 5, 10, 50 and 200 μ M rapamycin added. (C) Mean longevity values for the data in (A) and (B).

Supplementary Table

Table 1. Analysis of variance for effects of rapamycin and experimental block on *Drosophila* longevity

1% yeast	DF	L-R χ^2	P<	Term	Risk Ratio	Lower CL	Upper CL
Rapamycin	1	49.042	0.0000	Rapamycin 0uM	0.804	0.756	0.854
Block	1	0.057	0.8107	Block 2	1.007	0.948	1.071
Rapamycin*Block	1	5.907	0.0151	Rapamycin*Block	1.078	1.015	1.146
2% yeast	DF	L-R χ^2	P<	Term	Risk Ratio	Lower CL	Upper CL
Rapamycin	1	28.938	0.0000	Rapamycin 0uM	1.184	1.113	1.258
Block	1	43.275	0.0000	Block 2	1.227	1.155	1.304
Rapamycin*Block	1	8.339	0.0039	Rapamycin*Block	0.916	0.862	0.972
3% yeast	DF	L-R χ^2	P<	Term	Risk Ratio	Lower CL	Upper CL
Rapamycin	1	0.001	0.9696	Rapamycin 0uM	1.001	0.943	1.062
Block	1	0.728	0.3936	Block 2	0.975	0.918	1.034
Rapamycin*Block	1	10.882	0.0010	Rapamycin*Block	0.905	0.853	0.960
4% yeast	DF	L-R χ^2	P<	Term	Risk Ratio	Lower CL	Upper CL
Rapamycin	1	1.037	0.3084	Rapamycin 0uM	1.031	0.972	1.093
Block	1	24.081	0.0000	Block 2	0.861	0.812	0.914
Rapamycin*Block	1	12.613	0.0004	Rapamycin*Block	1.112	1.049	1.179

Proportional hazards analyses were conducted in the JMP statistical package. Two-way models were analyzed separately by diet (1, 2, 3, or 4% yeast), and results are shown in the left four columns. Risk ratio values are shown in the right four columns, with the reference conditions being 0uM Rapamycin and Block 2 for the two main effects. A lower risk ratio value means that treatment has a lower risk of death, or an extended longevity. These analyses seek to determine the direction and repeatability of the rapamycin effect on longevity. For yeast level of 1%, there is a highly significant rapamycin effect showing a reduced risk ratio (0.804) for the no-rapamycin control. The Block effect is not significant meaning that the two independent experiments gave similar results (the confidence limits (CL) of the risk ratio for Block in the 1% diet include 1.0, or no change in risk). The interaction term has a *P* value <0.05, but with 4 tests this is not significant as the critical value should be $0.05/4 = 0.0125$.

In the other diet levels (2, 3, and 4%), there is either a significant block effect or no significant rapamycin effect, indicating that the effects of rapamycin are not repeatable, or not measurable. The significant interaction terms for 2, 3, and 4% diets indicate that the difference in longevity between rapamycin and control treatments is significantly different in the two experimental blocks.